

## INVESTIGATION ON ANTIMICROBIAL EFFECTS OF SOME LICHEN SPECIES COLLECTED FROM KASTAMONU REGION

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### ABSTRACT

Lichens are symbiotic organisms of fungi and photobionts (algae or cyanobacteria). It has been known for many years that some secondary metabolites produced by lichens have antagonistic effects on different microorganisms, yeasts and algae.

In this study, *in vitro* antimicrobial activity of *Pseudevernia furfurace*, *Hypogymnia tubulosa*, *Evernia divaricata*, *Letharia vulpina* samples collected from Kastamonu region were examined against *Candida albicans* ATCC 26555, *Salmonella enterica* Serotype Typhium SL 1344, *Escherichia coli* ETEC LM 63083, *Shigella flexneri* and *Bacillus megaterium*. The antimicrobial activity of samples was evaluated by disc diffusion method and the results were supported by MIC (minimum inhibitory concentration) and MBC/MFC (minimum bactericidal/fungicidal concentration) tests.

According to the results, among the lichen samples, *Letharia vulpina* presented an antimicrobial activity against *B. megaterium*, where *Pseudevernia furfurace* had an antimicrobial activity against *S. flexneri* and *B. megaterium*, and *Evernia divaricata* against *C. albicans*, *S. flexneri* and *B. megaterium*. On the other hand, *Hypogymnia tubulosa* presented no antimicrobial activity.

**KEYWORDS:** Lichens, antimicrobial activity, disc diffusion method, MIC, MBC, MFC

## INTRODUCTION

Most of the plants have an almost limitless ability to synthesize aromatic substances, most of which are in phenolic nature or their derivatives (Geissman, 1963). These substances are often used as a defence mechanism against microorganisms, insects and herbivores (Samidurai and Saravanakumar, 2009).

Lichens are formed by a symbiotic relationship between algae or cyanobacteria, which is known as photobionts and fungi (Baron, 1999). In lichens individual photobiont cells are embedded in a tissue formed by the fungus (Brodo and Sharnoff, 2001). Lichens synthesize a variety of secondary metabolites, many of which are unique (Molnár and Farkas, 2010) and it is known for years that these metabolites have an antagonistic effect on different microorganisms, yeast and algae (Burkholder, Ans, McVeigh and Thornton, 1994). Therefore, lichens are used in medicine (Gucin, Ozturk, Dulger and Guvenc, 1997).

Substances presenting antimicrobial activity are not synthesized through their metabolism when algae and fungi isolated separately (Harmala, Hiltunen and Oksman, 1992; Lawrey, 1986). This shows the importance and necessity of symbiotic relationship in synthesizing antimicrobial substances in lichens (Gucin, Ozturk, Dulger and Guvenc, 1997). When algae or cyanobacteria and fungi are in symbiotic relationship, the secondary metabolites are synthesized by the fungi partner (Baron, 1999). Over 800 lichen metabolites have been identified (Huneck and Yoshimura, 1996), but aliphatic acids, pulvinic acid derivatives, depsides and depsidones, dibenzofuans, anthraquinones, naphthoquinones as well as epidithiopiperazinediones are most common secondary metabolites found in lichens (Müller, 2001).

The first study about the antimicrobial activity of lichens was conducted in 1944 (Burkholder, 1994). Today, more than 60 antibiotic substances are derived from lichens (Kotan, Alpsoy, Anar, Aslan and Agar, 2011). It is reported that the most active antimicrobial substances in lichens are usnic acid, pulvinic acid derivatives such as vulpinic acid and aliphatic acids which are especially active against gram positive bacteria and some fungi (Osmanağaoğlu, Yıldız and Sacılık, 2000; Vartia, 1973).

In addition to those, some other important properties of lichens are their anti-herbivore (Lawrey, 1984; Lawrey, 1986) and antitumor activities (Takai, Ueraha and Beisler, 1979).

In this study, *in vitro* antimicrobial activity of *Pseudevernia furfurace*, *Hypogymnia tubulosa*, *Evernia divaricata*, *Letharia vulpina* samples collected from Kastamonu region were examined against *Candida albicans* ATCC 26555, *Salmonella enterica* Serotype Typhimurium SL 1344, *Escherichia coli* ETEC LM 63083, *Shigella flexneri* and *Bacillus megaterium*.

## MATERIALS AND METHODS

### *Lichen Samples*

*Pseudevernia furfurace*, *Hypogymnia tubulosa*, *Evernia divaricata* and *Letharia vulpina* samples were collected from Kastamonu, TURKEY. The locations were given in Table 1.

**Table 1.** Localisations of the lichen samples

LICHEN SAMPLE	LOCATION
<i>Pseudevernia furfurace</i>	Kastamonu, Ilgaz Mountain, Derbent.
<i>Hypogymnia tubulosa</i>	Kastamonu, Küre Mountain, Çatak Dam.
<i>Evernia divaricata</i>	Kastamonu, Handüzü Forest.
<i>Letharia vulpina</i>	Kastamonu, Bostan Village, Aşağı Tüfekçi.

### *Extraction Solvent*

Altuner (2008) stated that sterile distilled H<sub>2</sub>O (sd H<sub>2</sub>O), ethanol, methanol, chloroform, benzene, diethyl ether and ethyl acetate can be used to extract active substances.

Cowan (1999) proposed that most of the active substances are in aromatic or saturated organic nature. Therefore, these substances can easily be extracted by ethanol or methanol. For this reason methanol (anhydrous) (Sigma-Aldrich, Germany) was chosen in the study.

### *Microorganisms*

*Candida albicans* ATCC 26555, *Escherichia coli* ETEC LM 63083, *Salmonella enterica* serotype Typhimurium SL 1344, *Shigella flexneri* (clinical isolate) and *Bacillus megaterium* (soil isolate) were used in the

study (Kastamonu University, Department of Biology, Botanical Research Laboratory Culture Collection).

### ***Preparation of Inocula***

All bacterial strains were incubated in atmospheric air at 37°C for 24 hours and *C. albicans* at 27°C for 48 hours. Inocula were prepared by transferring morphologically similar colonies of each organism into 0.9% sterile saline solution until the visible turbidity was equal to 0.5 McFarland standard having approximately  $10^8$  cfu.mL<sup>-1</sup> for bacteria and  $10^7$ cfu.mL<sup>-1</sup> for *C. albicans* (Hammer, Carson and Riley, 1999). Mueller-Hinton Agar (Merck, Germany) medium is used for bacteria, where *C. albicans* strain was plated on Sabouraud Dextrose Agar (Merck, Germany).

### ***Extraction Procedure***

Lichen samples were pulverised by using mortar and pestle. Pulverised samples were extracted in methanol by shaking at room temperature for 24 hours. Extracts were filtrated after 24 hours by using Whatman No 1 filter paper. Filtrates were evaporated by a rotary evaporator at 30°C and pulverised by a freeze-dryer. The pulverised residues were used to prepare extracts having 150 mg.mL<sup>-1</sup> concentrations.

### ***Disc Diffusion Method***

Disc diffusion test was performed as described previously by Andrews (2007). The culture medium was poured into 120 mm sterile Petri dish to give a mean depth of 4.0 mm ± 0.5 mm (Altuner and Çetin, 2009). 10 µl, 20 µl and 30 µl aliquots of each extract were applied on sterile paper discs of 6 mm diameter (Mahasneh and El-Oqlah, 1999). To get rid of any residual solvent which might interfere with the results, discs were left to dry overnight at 30°C in sterile conditions (Silici and Koc, 2006). The surface of the plates was inoculated using previously prepared inocula containing saline suspension of microorganisms. Inoculated plates were then left to dry for 5 minutes at room temperature before applying the discs. Discs were firmly applied to the surface of the plate which had an even contact with the agar. Plates were incubated and inhibition zone diameters were expressed in millimetres.

### ***Determination of MIC***

Broth dilution method for Minimum Inhibitory Concentration (MIC) determination as described in Basile et al. (1998) was performed. Serial 2-fold dilutions were made to obtain a concentration range of 0.0039 - 2 mg.mL<sup>-1</sup>. The MIC was defined as the lowest concentration of extract inhibiting any visible bacterial growth.

### ***Determination of MBC and MFC***

The Minimum Bactericidal Concentration (MBC) and the Minimum Fungicidal Concentration (MFC) determination were performed by sub-culturing suspensions from non-turbid MIC test tubes to agar medium. The MBC and MFC values were defined as the lowest concentration of extract inhibiting bacterial and fungal growth.

### ***Controls***

All extraction solvents and empty sterile discs were used as negative controls.

### ***Statistics***

The data determined as the mean of 3 parallel studies. All values given here are mean values of these 3 parallel studies.

The statistical analysis was performed using a non-parametric method Kruskal-Wallis one-way analysis of variance. A value of  $P < 0.05$  was considered statistically significant.

## **RESULTS**

The aim of this study was to investigate the antimicrobial activity of some lichen samples collected from Kastamonu region. To do this, the first test performed was disc diffusion test. In this test, extracts were loaded on empty sterile discs and these discs were then applied on a culture medium inoculated with microorganisms. If the extracts were active against microorganisms, they have caused an inhibition zone. The diameters of the inhibition zones recorded as the diameters of the zones in millimetres are given in Table 2.

Antimicrobial substances may have lethal or static type of activity. Lethal agents have a capability of killing microorganisms, where static agents have

a capability of inhibiting the growth or reproduction of microorganisms. The disc diffusion test alone is not enough to decide whether the activity type is lethal or static. In order to identify the type of the activity the disc diffusion test should be followed by MIC and MBC/MFC tests. Lethal agents have MFC values that are close to the MIC values. For static agents, the MIC values are much lower than the MFC values.

The MIC values, which were defined as the lowest concentration of extract inhibiting any visible microorganism growth stated as  $\text{mg.mL}^{-1}$  are given in Table 3. The MBC and MFC values which were defined as the lowest concentration of extract inhibiting bacterial and fungal growth after sub-culturing suspensions from non-turbid MIC test tubes to agar medium stated as  $\text{mg.mL}^{-1}$  are given in Table 3.

According to these results, *Letharia vulpina* showed antimicrobial activity on *B. megaterium*, *Pseudevernia furfurace* on *S. flexneri* and *B. megaterium*, and *Evernia divaricata* on *C. albicans*, *S. flexneri* and *B. megaterium*. On the other hand, *Hypogymnia tubulosa* presented no antimicrobial activity against any microorganism tested.

## DISCUSSION AND CONCLUSION

When disc diffusion test results are interpreted by using the MIC and MBC/MFC values, it can be concluded that the activity of *Evernia divaricata* on *C. albicans*, *S. flexneri* and *B. megaterium*, and the activity of *Letharia vulpine* on *B. megaterium* were lethal type of activity.

If Table 3 is examined, it can be stated that the activity of *Pseudevernia furfurace* on *S. flexneri* and *B. megaterium* was static type of activity.

But these results should be supported by further large-scale studies, which will emphasize mainly the characterization of antimicrobial agents and their mechanism of action.

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**Table 2.** Disc Diffusion Test Results (Inhibition zones - mm)

		<i>Pseudevernia furfuracea</i>	<i>Hypogymnia tubulosa</i>	<i>Evernia divaricata</i>	<i>Letharia vulpina</i>
<i>C. albicans</i>	10µl	-	-	-	-
	20µl	9	-	-	-
	30µl	12	-	-	-
<i>S. enterica</i>	10µl	-	-	-	-
	20µl	-	-	-	-
	30µl	-	-	-	-
<i>E. coli</i>	10µl	-	-	-	-
	20µl	-	-	-	-
	30µl	-	-	-	-
<i>S. flexneri</i>	10µl	8	12	-	-
	20µl	10	17	-	-
	30µl	11	22	-	-
<i>B. megaterium</i>	10µl	-	-	12	-
	20µl	9	-	18	-
	30µl	12	-	21	9

“- “no activity observed.

**Table 3.** MIC and MBC/MFC results (Active concentration - mg.mL<sup>-1</sup>)

	<i>Pseudevernia furfuracea</i>		<i>Hypogymnia tubulosa</i>		<i>Evernia divaricata</i>		<i>Letharia vulpina</i>	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
<i>C. albicans</i>	-	-	-	-	10	20	-	-
<i>S. enterica</i>	-	-	-	-	-	-	-	-
<i>E. coli</i>	-	-	-	-	-	-	-	-
<i>S. flexneri</i>	40	80	-	-	5	5	-	-
<i>B. megaterium</i>	20	40	-	-	5	10	40	40



“-“:no activity observed.

**ÖZET:** Likenler mantar ve alglerin oluşturduğu simbiyotik organizmalardır. Uzun yıllardan beridir, likenlerin ürettiği bazı sekonder metabolitlerin çeşitli mikroorganizma, maya ve algler üzerine antagonistik etki gösterdiği bilinmektedir. Bu çalışmada, Kastamonu yöresinden toplanan *Pseudevernia furfurace*, *Hypogymnia tubulosa*, *Evernia divaricata*, *Letharia vulpina* örneklerinin, *Candida albicans* ATCC 26555, *Salmonella enterica* Serotype Typhium SL 1344, *Escherichia coli* ETEC LM 63083, *Shigella flexneri* ve *Bacillus megaterium* üzerine *in vitro* etkileri çalışılmıştır. Örneklerin antimikrobiyal etkileri disk difüzyon metodu ile ölçülmüş olup, sonuçlar MİK (Minimum inhibisyon konsantrasyonu) ve MBK/MFK (minimum bakterisidal konsantrasyonu/minimum fungisidal konsantrasyonu) testleri ile desteklenmiştir. Sonuçlara göre liken örneklerinden *Letharia vulpine*, *B. megaterium* üzerine; *Pseudevernia furfurace*, *S. flexneri* ve *B. megaterium* üzerine; *Evernia divaricata*, *C. albicans*, *S. flexneri* ve *B. megaterium* üzerine antimikrobiyal etki gösterirken, *Hypogymnia tubulosa* herhangi bir etki göstermemiştir.

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